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(21) International Application Number: PCT/US90/03204 (22) International Filing Date: 6 June 1990 (06.06.90) (30) Priority data: 362,200 6 June 1989 (06.06.89) US (71) Applicant (for all designated States except US): NORTHWESTERN UNIVERSITY [US/US]; 633 Clark Street, Evanston, IL 60208 (US). (72) Inventor; and (75) Inventor/Applicant (for US only) : LETSINGER, Robert, L. [US/US]; 316 Third Street, Wilmette, IL 60091 (US). (74) Agent: KOHN, Kenneth, I.; Tilton, Fallon, Lungmus & Chestnut, P.O. Box 4390, Troy, MI 48099 (US).		(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent), US. Published <i>With international search report.</i>
(54) Title: STEROID MODIFIED OLIGONUCLEOTIDES (57) Abstract Oligonucleotides modified at their backbones by the attachment of at least one steroid are described. The modified oligonucleotides anchor in the cell membrane to serve as a probe and to provide therapeutic activity.		

* See back of page

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STEROID MODIFIED OLIGONUCLEOTIDES

GRANT REFERENCE

5 This invention was developed with support
provided by the National Cooperative Drug Discovery
Group for the Treatment of AIDS, Grant U01 A124846
from the National Cancer Institute of Allergy and
Infectious Disease and by Grant 5R37GM10265 from the
10 National Institute of General Medical Science.

FIELD OF INVENTION, BACKGROUND AND PRIOR ART

 This invention relates to oligonucleotides
15 modified by a pendant steroid group. More
particularly, the present invention is related to
steroid modified oligonucleotides and a method of
using the modified oligonucleotides as antiviral
agents.

20 The pioneering work of Zamecnik and
Stephenson, Proc. Natl. Acad., 75:280-284 (1978), on
antiviral activity of oligonucleotides and Miller and
Ts'o, on the chemistry and biochemistry of non-ionic
analogues (Barrett, et al., Biochem., 13:4898-5
25 (1974) and Jayaraman, et al. Proc. Natl. Acad. Sci.
USA, 78:1537-1541 (1981)) has stimulated extensive

- research directed at the therapeutic potential of nucleotide polymers. Oligonucleotide analogues with methylphosphonate, Miller, et al., Biochemie, 67:769-776 (1985), Agris, et al., Biochem., 25:6268-6275 (1986), Smith et al., Proc. Natl. Acad. Sci. USA, 83:2787-2791 (1986), and Sarin, et al., Proc. Natl. Acad. Sci. USA, 85:7448-7451 (1988); phosphorothioate, Matsukura, et al., Proc. Natl. Acad. Sci. USA, 85:7079-7083 (1988); and
- 10 phosphoramidate, Agrawal, et al., Proc. Natl. Acad. Sci. USA, 85:7079-7083 (1988), backbones as well as natural type oligonucleotides, Zamecnik, et al., Proc. Natl. Acad. Sci. USA, 83:4143-4146 (1986), and a polylysine conjugate, Goodchild, et al., Proc.
- 15 Natl. Acad. Sci. USA, 85:5507-5511 (1988), have now been reported to inhibit viral replication in cell culture. The viruses studied in this context include Rous sarcoma virus, Samecnik and Stephenson, Proc. Natl. Acad., 75:280-284 (1978); simian virus, Miller,
- 20 et al., Biochemie, 67: 769-776 (1985); vesticular stomatitis virus, Agris, et al., Biochem., 25:6268-6275 (9186) and Lemaitre, et al., Proc. Natl. Acad. Sci. USA, 84:648-652 (1987); human immunodeficiency virus (HIV), Sarin, et al., Proc. Natl. Acad. Sci.
- 25 USA, 85:7448-7451 (1988), Matsukura, et al., Proc. Natl. Acad. Sci. USA, 84:7706-7710 (1987), Agrawal,

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et al., Proc. Natl. Acad. Sci. USA, 85:7079-7083
(1988), Aamecnik, et al., Proc. Natl. Acad. Sci. USA,
83:4143-4146 (1986), and Goodchild, et al., Proc.
Natl. Acad. Sci. USA, 85:5507-5511 (1988); herpes
5 simplex virus, Smith, et al., Proc. Natl. Acad. Sci.
USA, 83:2787-2791 (1986); and influenza virus,
Zerial, et al., Nuc. Acids. Res., 15:9909-9919
(1987).

The concept underlying this work is that an
10 oligonucleotide complementary to a unique segment of
a viral genome, or an RNA derived from it, may
selectively disrupt processes dependent on that
segment by hybridization. This rationale is
supported by a variety of experiments with cell free
15 systems or with cells to which "antisense"
polynucleotides have been inserted by microinjection
or transfection, C.A. & Cohen, J.S., Cancer Res.,
48:2659-2668 (1988). However, the actual mechanisms
by which oligonucleotides and their analogs function
20 as inhibitors in cell cultures are still far from
clear. In particular, little is known about the
interaction of the oligomers with cell membranes or
the locus of their reactions within cells. It
appears that non-ionic oligomers, such as the methyl
25 phosphonate analogues diffuse passively through cell
membranes.

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S.E. Clare has synthesized oligonucleotides possessing one or more 2,2,2,-trichloro-1,1-dimethylethyl (TDCME, lipophilic) group of the phosphorous atom in the chain and show that this group on one strand with proper stereochemistry can inhibit cleavage of the opposite strand by a restriction endonuclease and that the same group on a template will inhibit synthesis of the complementary strand by the Klenow enzyme. S.E. Clare also demonstrated a single modification 5' to dGNAd (CG) octamer by TDCME group prevents the B to Z conformational transition. S.E. Clare, Ph.D. Dissertation, Northwestern University, Evanston, Illinois (1987).

The present invention is related to a family of oligonucleotides modified at the backbone so that the oligonucleotide may anchor at the cell membrane to provide antiviral effects. The present invention describes a family of oligonucleotides with a modification designed to anchor the oligomer, at least transiently at the cell membrane, to inhibit HIV-1 in cell culture. Fatty substances have been selected as an anchor for the oligonucleotide, and without being limitative, steroids such as cholesteryl have been selected as the preferred

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anchor since they are highly hydrophobic and cell membranes have an abundance of this steroid. The compounds may also have anti-sense activity.

The cholesteryl is a large lipophilic group, much larger than the TDCME group. In principle, such pendent groups, when linked covalently to the internucleotide phosphorous atoms, have potential as lipophilic centers to enhance the interaction with membranes, to alter partitioning of oligonucleotides within cells, to inhibit certain enzymatic reactions and to influence the stability of hybrids joined with natural polynucleotides.

Cholesteryl is a component of any biological membrane and interacts with other lipids. The AIDS virus, HIV, is distinguished by an unusually high cholesteryl content in the lipid membrane. Early model studies by Finean, Experientia, 9:17-19 (1985), suggested that the cholesteryl molecule is capable of formation of a stabilizing complex with the phospholipid molecule. The hydrocarbon chain of the cholesteryl is bound to the parallel portion of the phospholipid chain by Van DeWall forces. Recent studies employing a variety of techniques indicated that the major forces may involve the hydrophobic

portion of the lipid molecules. Therefore, cholesteryl is a preferred modifying group for oligonucleotide interaction with cells.

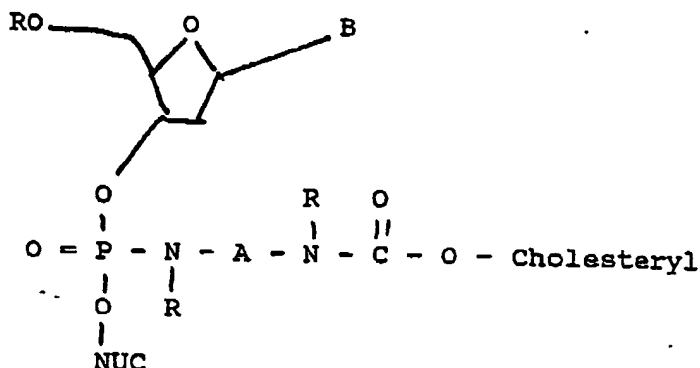
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SUMMARY OF THE INVENTION

The invention is concerned with oligonucleotides that are modified to anchor the oligomer at the cell membrane or eventually,
10 intranuclearly, so that the oligonucleotide may serve as a probe and provide therapeutic activity.

More specifically, the invention provides a pharmaceutical composition including an oligonucleotide conjugated to a steroid for
15 increasing the antiviral activity of the compound. One aspect of the present invention provides oligonucleotide compounds represented by the following structural formula:

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Formula I

wherein A = aliphatic alkyl or branched aliphatic alkyl or a heteroatom containing an alkyl (branched) chain of 2 to 18 carbon atoms, preferably CH_2 , R = H and lower alkyl up to 12 carbon atoms, preferably methyl; NUC refers to an oligonucleotide which may be a deoxyribonucleotide or a ribonucleoside. Preferred nucleotides are ethymidine, deoxyadenosine, deoxyguanosine and deoxycytidine. The nucleotides are connected respectively to the phosphorous through their 3' and 5' oxygens, B is a purine or pyrimidine base (such as Thy, Cyt, Gua, Ade).

Cholesteryl has been selected as the preferred anchol because it is highly hydrophilic and found in cell membranes. The cholesteryl modified oligonucleotides of the present invention have been

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found to inhibit HIV-1 in cell culture. The location of the insertion of the cholesteryl anchor on the oligonucleotide may be varied and is not dependent on sequence. However, other sterioids are efficacious

5

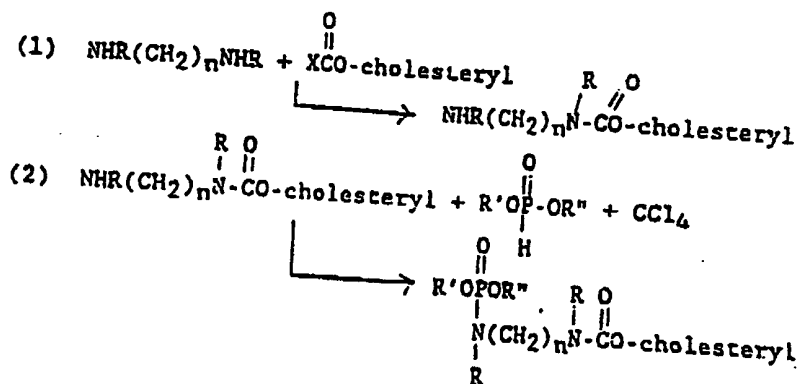
DETAILED DESCRIPTION OF THE INVENTION

The novel compounds of Formula I can be prepared by convenient procedures for introducing a cholesteryl group at any desired internucleoside phosphorous in the course of synthesizing an oligonucleotide. The cholesteryl may be linked to an oligonucleotide as a substituent at either the 3'-0 or 5'-0 terminus.

Processes for preparing the novel compounds of Formula I are generally described by equations A and B.

A.

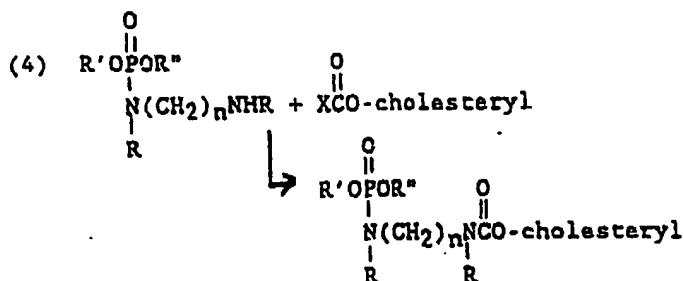
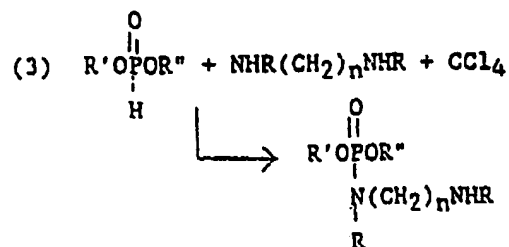
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B.



In the reactions, X = Cl- and p-nitrophenoxy, n = 2 and 6, and R = H and methyl. The reactions for linking the amines to phosphorous are based on the general procedure of Froehler, B.C., Tet. Lett., 27:5575-5578 (1986) for generating P-N bonds in oligonucleotide derivatives. The article is incorporated by reference.

Equation A avoids side reactions involving condensation at both nitrogen atoms of the diamine to form bis-phosphoramidates. Procedures for preparing fifteen compounds of Table I with the cholesteryl anchor are described as follows: phosphodiester links were formed by cyanoethyl phosphoramidite chemistry described in the standard synthesis protocol provided

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by the manufacturer of the synthesizer, for example, Biosearch 8600, Biosearch, Inc., San Raphael, California. Chain extension by hydrogen phosphonate chemistry is described by Froehler, et al., Tet. Lett., 27:469-472 (1986) and Froehler, et al., Nuc. Acids. Res., 14:5399-5407 (1986). Phosphorothioate functional groups are added by the procedure of Froehler, et al., Tet. Lett., 27:5575-5578 (1986).

10

Experimental Procedure2-(Cholesteryloxycarbonylamino)ethylamine.

Cholesteryl chloroformate (2g) in dichloromethane (6 ml) was added dropwise to a solution of ethylenediamine (2.5 ml) in dichloromethane (6 ml) and pyridine (6 ml). The mixture was stirred for two hours; then the solvent was removed under vacuum and the residue was partitioned between water (150 ml) and dichloromethane (150 ml). The organic layer was washed with water, dried (Na_2SO_4), and concentrated to give the title compound; 1.6 g (76%), mp 149-155 degrees Centigrade. Recrystallization from cyclohexane afforded crystals melting at 152-155 degrees Centigrade; Rf on silica ($\text{CHCl}_3/\text{MeOH}$, 1/1

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v/v) 0.15; positive ninhydrin test. Anal. Calcd for $C_{30}H_{52}N_2O_2$; C, 76.22; H, 11.09; N, 5.93. Found: c, 75.96; H, 11.10; N, 5.86.

5 Preparation of Cholesteryl-Modified Dinucleoside Monophosphate on CPG Support.

Internucleoside cholesteryl side chains were linked to phosphorous via phosphoramidate bonds (adaption of procedure of Froehler). The preparation of d-DMT-ibG_cibG-CPG. is representative. A sample of DMT-ibG linked through the 3'-O to a controlled-por-glass support (Biosearch) (250 mg. 8 micro moles of DMG-ibG) was placed in a Glencoe Gastight syringe (10 ml) equipped with a plug of glass wool at the inlet.

15 Reactions and washings were carried out by drawing in and ejecting the desired solutions. Thus, the DMT groups were removed with dichloroacetic acid (2.5% in CH_2Cl_2 ; the support was washed repeatedly with C_5H_5/CH_3CN (1/4), coupling was effected by drawing in

20 together DMT-ibG-hydrogen phosphonate (80 mg., 0.1 mmol, in 1.2 ml CH_3CN/C_5H_5) and trimethylacetyl chloride (65 micro L, 0.5 micro moles, in 1.2 ml CH_3CN/C_5H_5 , 1/1 v/v; 2 minutes), and the support was washed well with CH_3CN/C_5H_5 . A solution of

25 cholesteryloxycarbonylaminoethylamine (250 mg, 0.5 mmol) in CCl_4 (5 ml) and C_5H_5 (2 ml) was then drawn

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into the syringe and after 0.5 hours, the solution was ejected and the solid was washed well with CH_3CN . Appropriate portions were then transferred to a cartridge for extension by machine synthesis
5 (Biosearch 8600 Synthesizer) or to a syringe for manual synthesis.

Chain Extension. The oligonucleotide chains were extended by conventional phosphoramidite chemistry in constructing phosphodiester links and by
10 hydrogen phosphonate chemistry in building the phosphorothioate derivatives. The manual procedure used in adding a thymidine unit to DMT-ibG*G-CPG in synthesizing compound 2 in Table 1 is representative of one synthetic cycle utilizing a phosphoramidite
15 reagent.

The DMT(G*G) loaded CPG (30 mg, 1 micro mole) was poured into a 1.0 ml Glenco Gas tight syringe with a glass wool plug at the inlet. Washes were effected by drawing up the desired amount of the
20 reagent, resuspending the support by brief hand agitation, and ejecting the solution. The DMT protecting group was removed by washing with $\text{DCA}/\text{CH}_2\text{Cl}_2$ (2.5/100 v/v, 5.0 ml), and organ effluents were pooled from subsequent spectroscopy (447 nm) and
25 calculation of the coupling efficiency. The support was washed successively with $\text{C}_5\text{H}_5\text{N}/\text{CH}_3\text{CN}$ (1/4, v/v, 1

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- X 0.5 ml), and CH_3CN (2 X 0.5 ml). Any unreacted 5'-OH groups were capped by drawing DMAP in $\text{C}_5\text{H}_5\text{N}/\text{THF}$ (0.3 M, 1/15, v.v, 0.5 ml) into the syringe followed immediately by $\text{Ac}_2\text{O}/\text{THF}$ (0.6 M, 0.5 ml). The mixture
- 5 was agitated for one minute, capping agents were ejected from the syringe, and the support was washed with $\text{C}_5\text{H}_5\text{N}/\text{CH}_3\text{CN}$ (1/4, v/v, 1 X 5.0 ml) and CH_3CN (1X 0.5 ml). The phosphite internucleoside linkage was oxidized to the phosphotriester linkage with I_2 in
- 10 $\text{C}_5\text{H}_5\text{N}/\text{THF}/\text{H}_2\text{O}$ (0.1 M I_2 , 18/80/2, v/v/v, 0.5 ml) for two minutes. The oxidant was ejected, and the support was washed with $\text{C}_5\text{H}_5\text{N}/\text{CH}_3\text{CN}$ (1/4, v/v, 3 X 1.5 ml) and CH_3CN (3 X 1.5 ml) to complete one synthetic cycle.
- 15 For chain extension by hydrogen phosphonate chemistry the DMT-ibG*ibG-cpg (1 micro mole loaded dimer) was detritylate as in the previous case. A solution of the DMT-nucleoside hydrogen phosphonate (10 mg, about 15 micro moles, in $\text{CH}_3\text{CN}/\text{C}_5\text{H}_5\text{N}$ (1/1,
- 20 v/v, 0.3 ml) was drawn into the syringe, which was agitated for two minutes. The coupling agents were ejected from the syringe, and the support washed with $\text{C}_5\text{H}_5\text{N}/\text{CH}_3\text{CN}$ (1/1, v/v, 0.5 ml), and CH_3CN (3 X 0.5 ml) to complete one synthetic cycle. Additional
- 25 couplings were performed by returning to the initial wash and repeating the cycle. Oxidation following

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the final coupling step was performed by treatment with 0.1 M sulfur in $\text{CCl}_4/\text{Et}_3\text{N}$ (9/1, v/v) at room temperature (two hours reaction). Procedures for machine syntheses were similar.

5

Isolation of Oligonucleotides

The oligomers were removed from the syringe or the synthesizer and warmed in a capped vessel with concentrated NH_4OH at 55 degrees Centigrade for five
 10 hours. The aqueous solution was then removed and concentrated under reduced pressure to give the crude oligonucleotide. This substance was chromatographed on a C-18 column and the band corresponding to the
 15 desired target oligomer was collected and lyophilized.

Table 1. Properties of Oligonucleotides

compound		HPLC ^a min	TLC ^b R _f	PAGE ^c R _m	T _m ^d °C	
20	1	ACAGCGAATTCGAAAATGC	12.2	0.26	0.64	60
		Cholesteryl Substituents				
	2	ACACCGAATTCGAAAATG*G	46.0	0.41	0.55	60
	3	ACTCCGAAAGATAAAG*G	46.6	0.43	0.54	--
	4	A*CACCGAATTCGAAAATG*G	61.0	0.58	a	52
	5	CAATTCGAAAATG*G	46.5	0.54	0.64	46.5
	6	A ₃ C ₂ A ₂ C ₂ C ₂ C ₂ A ₂ T ₂ T ₂ C ₂ T ₂ G ₂ A ₂ A ₂ A ₂ T ₂ G ₂ C	16.5	0.59	0.67	44
	7	A ₃ C ₂ A ₂ C ₂ C ₂ C ₂ A ₂ T ₂ T ₂ C ₂ T ₂ G ₂ A ₂ A ₂ A ₂ T ₂ G*G	40.2	0.61	0.64	47.5
	8	C ₂ A ₂ A ₂ T ₂ T ₂ C ₂ T ₂ G ₂ A ₂ A ₂ A ₂ T ₂ G ₂ G	17.3	0.47	0.67	29
	9	C ₂ A ₂ A ₂ T ₂ T ₂ C ₂ T ₂ G ₂ A ₂ A ₂ A ₂ T ₂ G*G	47.4	0.59	0.63	25
25	10	C ₂ T ₂ G ₂ A ₂ A ₂ A ₂ T ₂ G*G	15.6	0.52	0.76	--
	11	C ₂ T ₂ G ₂ A ₂ A ₂ A ₂ T ₂ G*G	49.6	0.56	0.62	--
	12	C ₂ A ₂ C ₂ T ₂ T ₂ T ₂ T ₂ A ₂ G*G	45	0.60	0.79	--
	13	C ₂ T ₂ G ₂ A ₂ T ₂ T ₂ T ₂ T ₂ G*G	45.2	0.59	0.76	--
	14	A ₂ A ₂ A ₂ G*G	47.5	0.66	0.62	--
	15	T ₂ T ₂ T ₂ T ₂ T ₂ T ₂ T ₂ T ₂ T ₂ T ₂ T ₂ T ₂ T*T	50.0	0.60	0.65	16

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In formulas * represents $\text{O}=\text{P}-\text{NH}(\text{CH}_2)_2\text{NHCO}_2$ Cholesteryl; s represents $\text{O}=\text{P}-\text{S}^-$, and + represents $\text{O}=\text{PNH}(\text{CH}_2)_3\text{N}(\text{CH}_3)_2$. Altered nucleotides in 7 are underlined.

5 ^aElution time, Hewlett-Packard RP-C18 column (10 cm); 0.1 M triethylammonium acetate (pH 7.0), 1%/min acetonitrile gradient starting at 0% acetonitrile; 0.5 ml/min flow rate.

^bThin layer chromatography on Merck silica
10 plates with propanol/ammonium hydroxide/water, 55/10/35 v/v/v.

^cPolyacrylamide gel electrophoresis in 20% crosslinked gel at pH 8.0, R_m is migration relative to bromophenol blue.

15 ^d T_m is the temperature at the midpoint for the maximum slope in a plot of A_{260} versus temperature in 0.1 M aqueous NaCl, 0.01 M Tris buffer at pH 7.0; total nucleotide concentration (base units) in approximately 10^{-4} M. In each case, the
20 complement is a phosphodiester strand equal in length to the modified oligomer.

^eThe sample appeared as a broad streak starting at R_m 0.2. The complement for determination of T_m was poly d(A).

25

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Coupling efficiency introducing a cholesteryl fragment to the compounds of Table 1 exceeded 50%.

As noted, phosphodiester links were formed in compounds 2-5 by conventional cyanoethyl phosphoramidate chemistry as described in the standard synthesis protocol provided by the manufacturer of the synthesizer, Biosearch, Inc., San Raphael, California. For compounds 6 - 15, the chains were extended by hydrogen phosphonate chemistry as described by Froehelr, et al., Tet. Lett., 27:469-472 (1986) and Froehler, et al., Nuc. Acids. Res., 14:5399-5407 (1986) the final oxidation with sulfur to generate the phosphorothioate groups.

The compounds were characterized by HPLC, TLC, PAGE, thermal disassociation curves for hybrids formed with complimentary strands and by UV and NMR spectroscopy. The NMR spectra exhibited the characteristic peaks for phosphodiester, phosphoramidate and phosphorothioate functional groups. A proton NMR spectrum of compound 2 shows the presence of the cholesteryl fragment. Further, the hydrophobic nature of the cholesteryl-oligonucleotides was shown by the HPLC data in Table 1. For example, the elution times for samples analyzed by a reverse phase C-18 column increased

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from 14 to 46 to 61 minutes for the series 1 (control), 2(one) cholesteryl, and 4(two) cholesteryl, respectively. Susceptibility to nuclease degradation was examined with compound 2.

- 5 In the presence of snake venom, phosphodiesterase, an alkaline phosphatase, compound 2 was completely hydrolyzed to the expected nucleosides and the fragment corresponding to the terminal G*G.

- The data in Table 1 shows that the
- 10 introduction of a single cholesteryl fragment at a terminal internucleoside position has only a minor effect on the stability of the hybrid duplex as measured by T_m values when compound 1 is compared with 2; compound 6 compared with 7, and compound 8
- 15 compared with 9. Conversely, two cholesteryl substituents led to appreciable destabilization for the 20-mer compound, compare compound 4 with 1. The disassociation of complexes formed from equi-molar quantities in modified and unmodified complementary
- 20 oligodeoxyribo-nucleotides were measured by changes in adsorbents in 260 nm as a function of temperature.

- Additional compounds were made in accordance with Equation B, wherein $R = CH_3$, $N = 6$, $X = -OC_6H_5MO_2$. The following compounds were prepared:
- 25 16, TTTTTTTTT#T; 17, T#TTTTTTTTT; 18, TTTT#TTTTTT; and 19, CGCG#AATTCGCG, where # is $O = P-N$

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(CH₃)(CH₂)CO₂ cholesteryl. The procedures of Froehler, B.C., Tet. Lett., 27:5575-5578 (1986) and Marcus=Sekura, et al., Nuc. Acids. Res., 15:5749-5763 (1987) were followed in preparing phosphodiester

5 links in compounds with internal modifications to avoid or minimize complications which could arise from the premature formation of phosphodiester groups in the course of the synthesis. As in the case of the derivatives of ethylenediamine, introduction of

10 substituents at the terminal internucleoside links had little effect on T_m values for the hybrids formed in complementary sequence (T_m for 1:1 complexes with poly d(A) in 0.1 M NaCl, pH 7: 28 degrees Centigrade for compound 16, 27 degrees Centigrade for compound

15 17 and 28 degrees Centigrade for parent T₉T). Conversely, modification of the centrally positioned internal link led to significant destabilization (21 degrees centigrade T_m for compound 18). The mobility of stereoisomers of compound 19 on HPCL differed

20 sufficiently to permit separation of the isomers. The T_m for the duplexes formed from these self-complementary modified strands (stereoisomers of compound 15) were substantially lower than that for the parent duplex (T_m:40 degrees and 45 degrees

25 Centigrade for the isomers as compared to 56 degrees Centigrade for CGCGAATTCGCG; 0.1 M NaCl, pH 7.0).

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The linking of cholesteryl to an oligonucleotide as a substituent at the 5'-O terminus is quite simple and can be shown by the following example.

5

Synthesis and characterization of cholesteryl-sTsGsG.

DMT(G) loaded CPG (88.2 mg, about 3 micro moles) was poured into 5 ml Glencoe gas tight syringe with a glass wool plug at the inlet. Washes were
10 effected by drawing up the desired amount of the reagent, resuspending the support by brief hand agitation and ejecting the solution. The support was initially washed with CH_3CN (4.5 ml X 3) and CH_2Cl_2 (4.5 ml X 2). The DMT protecting group was removed
15 by washing with $\text{DCA}/\text{CH}_2\text{Cl}_2$ (2.5/100, v/v, 10 ml). All of the orange effluents were pooled for subsequent spectroscopy (448 nm) and calculation of the coupling efficiency.

The support was washed successively with
20 pyridine/ CH_3CH (1/4, v/v, 4.5 ml X 3), CH_3CN (4.5 ml X 4) and dry CH_3CN (4.5 ml X 6). The H-phosphonate solution (for G and T - 36 mg, about 0.06 micro moles in dry $\text{CH}_3\text{CH}/\text{pyridine}$, 1/1, v/v, 2ml); for
cholesteryl H-phosphonate-42 mg., about 0.09 micro
25 moles in dry $\text{CH}_3\text{CN}/\text{pyridine}$, 1/3, v/v, 2.0 ml) and trimethylacetyl chloride solution (0.03 ml, about

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0.351 micro moles in trimethylactyl chloride solution (0.03 ml, about 0.351 micro moles in dry CH_3CN /pyridine, v/v, 2 ml) were drawn into the syringe, which was agitated for five minutes (but for 5 15 minutes for cholesteryl H-phosphonate coupling). After each coupling, the reagents were ejected from the syringe and the procedure was continued by returning to the initial wash steps.

Oxidation, following final coupling step and wash with dry CH_3CN (4.5 ml X 3), dry pyridine (4.5 ml X 4) was performed with 0.1 58 in CS_2 /pyridine (1/1, v/v, 4.5 ml X 2), CH_3N (4.5 ml X 3), dry CH_3CN (4 ml X 3) and ether (5 ml X 4). After drying the CPG-bound product was treated with 3.0 ml 15 concentrated NH_4OH at 55 degrees Centigrade for five hours. Upon removal of NH_4OH by evaporation under reduced pressure, CPG support was removed by filtration; the filtrate freeze-dried overnight and the product redissolved in 2.0 ml H_2O . For UV 20 spectroscopy, 10 micro liters of this solution were added to 990 micro liters of H_2O .

HPLC data indicated about 50% of the reaction mixture was the desired product. Spectroscopic methods also confirm the desired 25 structure.

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Compound of Formula I of the present invention can also be utilized in a method for hybridizing with a complementary sequence in a solution under conditions conducive to the hybridization. Typically, these conditions are controlled by the complementary sequence. The specific conditions needed for hybridization would be known to one skilled in the art familiar with the complementary sequence and environment for hybridization.

The present invention further comprises the method of modifying the backbone of an oligonucleotide by the attachment of a fatty substance, preferably cholesteryl so that it will anchor into the cell membrane so that the modified oligonucleotide will hybridize with the complementary sequence. By anchoring into the cell membrane, the oligonucleotide may provide diagnostic or therapeutic activity. For example, the oligonucleotide compound 1 of Table 1 is complementary to the splice acceptor for site at 5349-5368 in HIV-1 and has been shown to inhibit replication of this virus in MOLT-3 cells by Zerial, et al., Nuc. Acids Res., 15:9909-9919 (1987) and Stein, et al., Cancer Res, 48:2659-2668 (1988). The compounds shown in table 1 are structural variations of the basic sequence of compound 1.

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Compounds 2 - 15 were designed to provide information on the antiviral properties of the cholesteryl modified oligonucleotides and, specifically, on the dependence of the antiviral activity on 1 (the number of cholesteryl fragments incorporated in the backbone chain); 2 (the nature of the main backbone section e.g. phosphodiester versus phosphorothioate links), 3 (the length of the oligonucleotide) and 4 (the sequence integrity of the oligonucleotide).

10 Samples of the oligomers were assayed in the following test:

Assays for HIV-1 Inhibition.

 The inhibition of HIV-1 expression of H9 or
15 MOLT-3 cells in the presence of antisense oligonucleotides was carried out by infecting 5×10^5 cells per ml with $2.5-5 \times 10^8$ virus particles of HIV-1 strains HTLV-IIIB or HTLV-IIIC. Infection with 500-1000 virus particles per cell represents a
20 multiplicity of infection (MOI) of 0.5-1. HIV-1 infection of cells was carried out by simultaneous addition of virus and cholesteryl modified oligomers to the cells in culture. The cultures were incubated in culture medium containing RPMI 1640, 10% (v/v)
25 fetal bovine serum, 2mM glutamine, and 250 micrograms of gentamicin per ml, in a humidified atmosphere

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containing 5% CO₂ at 37 degrees Centigrade. After four days, the cells and supernatant were examined for the level of HIV-1 expression by measuring syncytia (MOLT-3 cells) and viral antigen expression as well as cell viability. The number of syncytia formed MOLT-3 cells were counted after triturating the cells to obtain an even distribution of the syncytia in the culture. The average number of syncytia as obtained by counting several fields in duplicate cultures. Cell viability was measured in the presence of trypan blue, and the cells that excluded the dye were counted as viable cells. HIV-1 antigen expression was measured in cells fixed in methanol/acetone as described. Sarin, et al.,
15 Biochem. Pharmacol., 34:075-4078 (1985) and Sarin, et al., J. Natl. Cancer Inst., 78:663-666 (1987). In brief, the cells were pelleted and then resuspended in phosphate-buffered saline (PBS) at a concentration of 10⁶ cells per ml. The cells were spotted on
20 toxoplasmosis slides, air-dried, and fixed in methanol/acetone (1:1, v/v) for 15 minutes at room temperature. The slides were next incubated with 10% normal goat serum at room temperature for 30 minutes and washed with PBS four times. HIV-1 p24 or P17
25 monoclonal antibody was added to each well and the slides were incubated for 30 minutes in a humid

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chamber 15 37 degrees Centigrade. The slides were then washed four times with PBS, incubated with fluorescein isothiocyanate-labeled goat anti-mouse IgG (Cappel Laboratories, Cochranville, Pennsylvania) 5 for 30 minutes at 37 degrees Centigrade, and then washed with PBS overnight. The slides were counterstained with Evan's blue, washed with PBS, mounted with 50% glycerol, and examined with a Zeiss fluorescence microscope. The percentages of cells 10 fluorescing in the oligomer-treated and untreated cultures were compared. Inhibition of HIV-1 expression in the presence of oligomers was found to be similar in both the H9 and the MOLT-3 cells.

Inhibition of HIV-1 expression and H9 and 15 MOLT-3 cells in the presence of cholesteryl modified oligonucleotides was carried out and results shown in Tables 2 and 3.

The data for the inhibition of formation of syncytia, an expression of HIV proteins P17, P24 and 20 reverse transcriptase shown for compounds 1 - 5 in Table 2 and compounds 6 - 16 in Table 3. The tables show results in ID_{50} values for inhibition of syncytia (concentration of an oligomer in micrograms/ml. It gives 50% inhibition under the assay 25 condition) as an index.

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The data from the tables describe favorable conclusions. The activity of the parent oligonucleotide, compound 1, is relatively low (ID50 less than 100). It appears that anchoring a cholesteryl fragment to the oligonucleotide significantly enhances the anti-HIV activity (from ID50 greater than 100 to 10). Thus, the cholesteryl provides steroid means conjugated to the oligonucleotide for increasing the antiviral activity of the oligonucleotide. Further, anchoring a second cholesteryl fragment does not appear to be an improvement because the second cholesteryl leads to a reduction in activity relative to the monocholesteryl-oligonucleotide. It appears that a cholesteryl fragment to a phosphorothioate oligonucleotide analog enhances the antiviral property of the phosphorothioate derivative as shown in comparisons between compounds 6 and 7, 8 and 8, and 10 and 11. In the most favorable case, compound 7, the ID50 was reduced to 0.8 micrograms per milliliter. With relatively large oligomers, those having 15 to 20-mers, the activity of the cholesteryl-oligonucleotides (natural phosphodiester links) appears to be independent of the chain link (compare compounds 2 and 5). A lack of dependence of activity on link has also been shown for unmodified

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oligonucleotides in the 15-20 mer range.

Additionally, the activity of the cholesteryl modified phosphorothioate derivatives shows a downward trend as the length of the oligomer is decreased.

5 Thus, The ID50 values increase from 0.8 for the 20-mer (compound 7) to about 3.5 for the 10-15 mers (compound 11 and 9), to 13 for the 6-mer (compound 14).

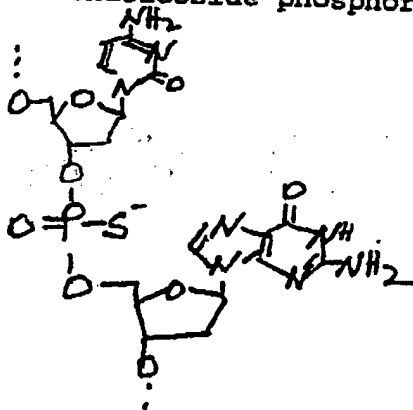
From Tables 2 and 3, it can also be
10 concluded that the anti-HIV activity of the cholesteryl-modified oligonucleotides is not strongly dependent on the nucleotide sequence. This conclusion applies both to the phosphodiester and the phosphorothioate cholesteryl derivatives (compared to
15 the data for compounds 2 and 3 has six mismatched base sites; and the data for compound 11 with that for compounds 12 and 13 which have 8 and 3 mismatched). For phosphorothioate derivatives, the activity of all three 10-mers is essentially the same
20 although the sequence is different. Further, the cholesteryl modified oligomers are not toxic to cells even at concentrations far in excess of those that lead to complete inhibition of the replication of HIV. For all derivatives the LB50 was greater than 100
25 micrograms per ml.

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Additional Oligonucleotides having Steroids
Conjugated Thereto Possessing Antiviral Activity

Additional compounds and their antiviral activity are set forth in Table 4. Activity is reported as the average ID50 values (the concentration in micrograms per ug/ml of oligomer that leads to 50% inhibition of virus) determined by syncytia formation and expression of the viral proteins P17 and P24. The procedure is referenced in Letsinger et al (1989) Proc. Natl. Acad. Sci. USA, 86:6553-6556. The abbreviations used in the Table are set forth below.

15 ...NsN'... internucleoside phosphorothioate link, as in ..CsG..

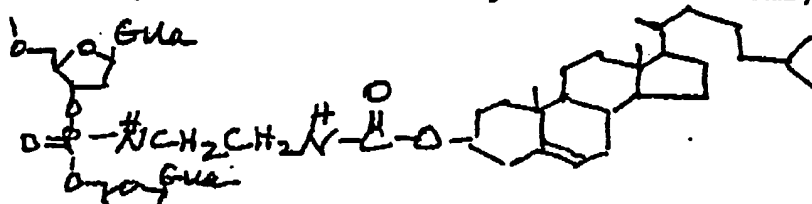


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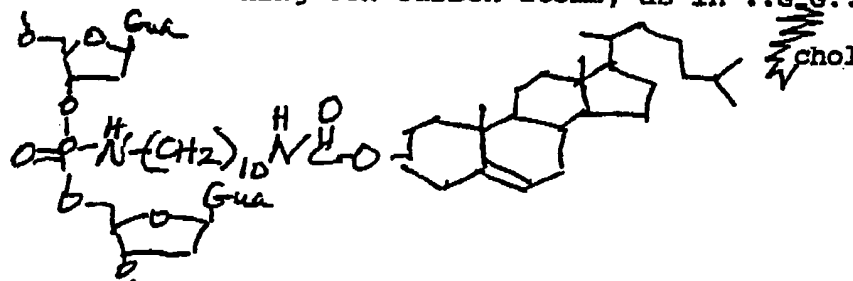
25

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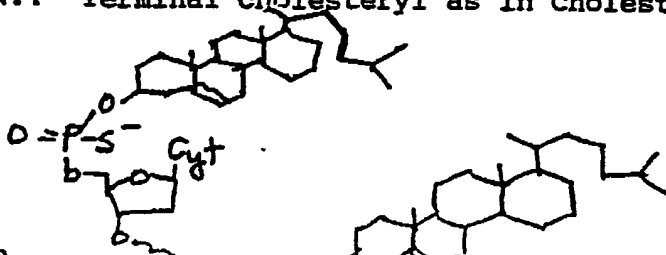
...N,N'... internucleoside link with a cholesteryl group tethered
 Zchol by a linker containing two carbon atoms, as in ..G.G..
 chol



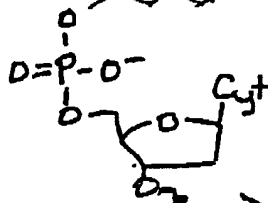
...N,N'... internucleoside link with a cholesteryl group tethered
 by a linker containing ten carbon atoms, as in ..G.G..
 chol



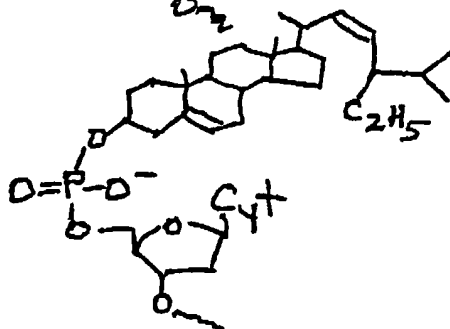
CholesterylSN... Terminal cholesteryl as in CholesterylSC...



CholesterylPC...



StigmasterylSC...



EXPERIMENTATION

All compounds were synthesized on controlled pore glass supports using a Biosearch 8600 DNA synthesizer or a syringe for manual manipulation. Internucleoside phosphorothioate links in compounds 1-4, 6, 8-16 were generated by conventional hydrogen phosphonate chemistry using 5'-dimethoxytritylnucleosides and terminal oxidation of all hydrogen phosphonate links with sulfur. Cholesteryl was joined to the chain in 1-4, 8, 10, 12, 14-16 by oxidative coupling of 2-(cholesteryloxycarbonylamino)-ethylamine at an internucleoside hydrogen phosphonate link via reaction with carbon tetrachloride. The procedure for tethering cholesteryl in compounds 11 and 13 was identical except that 2-(cholesteryl-oxycarbonylamino)-decylamine was used as the amine component. All these procedures are described in the original patent application and/or reference 1. For synthesis of 6, cholesteryl was joined utilizing cholesteryl H-phosphonate. For the synthesis of compounds with phosphodiester links 5' to phosphorothioate links (compounds 5 and 7), chains were built using methyl phosphoramidate coupling followed by oxidation with sulfur at each step. W. J.

Stec, et al, Am. Chem. Soc., 106:6077 (1984). The terminal cholesteryl phosphate was added by oxidative coupling with cholesteryl H-phosphonate and carbon tetrachloride, as for compound 6, followed by

5 standard oxidation with iodine/water. Treatment with thiophenol to remove the methyl protecting groups and ammonium hydroxide to remove base protecting groups and cleave the oligomer from the solid support the afforded the modified oligonucleotide.

Compounds 15, 16 and a related substance, compound 17 with the structure $XsXsXsXsXsXsXsXsX$ chol T, were prepared using the syringe technique and DMT- $OCH_2CH_2CH_2OP(O)(H)O^-$ (compound 18 in place of a nucleoside H-phosphonate. For synthesis of 18, 1,3-propanediol (75 mmol) was stirred with dimethoxytrityl chloride (15 mmol) in dry pyridine (25 ml) for about 20 hours at room temperature. Addition of water (100 ml), extraction with chloroform, and chromatography on a silica gel column afforded 3-dimethoxyltrityloxypropanol as a viscous oil; 56% yield; NMR in $CDCl_3$: 1.85 (p, 2H, $-CH_2-$), 2.21 (broad t, 1H, O-OH), 3.28 (broad t, 2H, $-CH_2(OH)$), 3.79 (broad t, 8H, two CH_3O-), 6.82 (q, 4H), 7.21-7.42 (m, 9H aromatic). This alcohol (6.9 mmol), in dry acetonitrile (100 ml) was added dropwise over a period of 1 hour to phosphitilating solution

prepared from phosphorus trichloride (30 mmol),
imidazol (98 mmol), and triethylamine (104 mmol) in
acetonitrile (100 ml) at ice-bath temperature. After
one hour of stirring the flask was allowed to warm to
5 room temperature and to stand for two hours. Water
(50 ml) was added and the mixture was concentrated
under reduced pressure and extracted with chloroform.
Chromatography on silica gel afforded 20.0 g (62%) of
18 as the triethylammonium salt; mp 132-135°C; NMR
10 (CDCl₃): ppm 1.22 (t, 9H), 1.91 (p, 2H), 2.93 (q,
6H), 3.13 (t, 2H), 3.72 (s, 6H), 3.97 (t, 2H), 6.05
and 7.57 (singlets each, 1H), 6.77 (q, 4H), 7.14-7.39
(m, 9H).

Referring to Table 4, compound 1 is
15 complimentary to a region in HIV-I coating for a
splice site for the TAT gene. Compound 3 is
complimentary to a region for REV gene. Compound 4
is complimentary to a region for NEF gene.

Compounds 2, 5, 6, 7, 8 and 9 are all based
20 on sequence 1, with variations in position, mode of
attachment, and the structure of the lipophilic
steroid conjugate.

Compounds 10-14 are conjugates of
homopolymers of nucleotides.

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Referring to the activities shown in Table 4, compounds 1, 3, and 4, which are complimentary to various regions of the HIV-I virus, all show unusually high antiviral activity. These compounds may function by an antisense mechanism. Compound 3 is the most active cholesteryl-conjugated oligonucleotide that has been investigated.

Oligonucleotide phosphorothioate derivatives bearing two cholesteryl groups, one tethered at an internal position, are active, as demonstrated by compounds 2 and 14. Activity for an oligonucleotide with cholesteryl groups tethered at the 5' and 3' terminal internucleoside positions as set forth above it has been reported.

Cholesteryl conjugates with mixed phosphorothioate and phosphodiester backbones are active as demonstrated by compounds 2, 5, and 7. As shown by compounds 5, 6, and 7, oligomers with cholesteryl attached at the 5' end, either by a phosphodiester or a phosphorothioate link, are active. The activity is not strongly dependent on which of these links is used; that is, ID50 is 7 ug/ml for the phosphorothioate, compound 6, while is it 12 ug/ml for the phosphodiester, compound 7.

Linkage by the carbonylaminoethylamidate, such as in compound 8, affords a more active compound than linking by a phosphodiester, such as compound 7, or a phosphorothioate, such as compound 6.

5 Other highly lipophilic groups, such as stigmasteryl (compound 9) are comparable to cholesteryl in enhancing the antiviral activity of the oligonucleotide derivatives.

 Cholesteryl conjugated homooligomers
10 containing thymidine, such as compounds 10, and 11, or deoxycytidine, such as compounds 12, and 13, are active antiviral pharmaceutical compositions. The deoxycytidine oligomers are somewhat active than the thymidine derivatives.

15 Tethers between the steroid and the oligonucleotide having long carbon chains of for example 10 methylene groups, as well as short carbon chains of 2 methylene groups can be used in linking the cholesteryl to the phosphorus atom, as shown by
20 comparing data for the thymidine derivatives (compounds 10 and 11) and the deoxycytidine derivatives (compounds 12 and 13. The compounds with the linker $\text{-NH(CH}_2\text{)}_{10}\text{NHCO-}$ exhibit about the same activity as the corresponding compounds with the
25 linker $\text{-NH(CH}_2\text{)}_2\text{NHCO-}$.

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In view of the above, applicant has demonstrated the antiviral activity of the pharmaceutical composition of an oligonucleotide conjugated to a steroid. Applicant has shown that
5 the oligonucleotide can be a homooligomer as well as a specific nucleotide sequence complimentary to various regions of a virus, such as the HIV-I virus. Applicant has also shown that various steroids conjugated to the oligomer have antiviral activity.

10 The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

15 Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims wherein reference numerals are merely for
20 convenience and are not to be in any way limiting, the invention may be practiced otherwise than as specifically described.

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Table 2. Inhibition of HIV by Oligonucleotides with Cholesteryl Substituents

compound	conc. μg/ml	% inhibition			ID ₅₀ μg/ml (syncytia)
		syncytia	P24	RT	
1 (control)	0.16	0			> 100
	0.8	3			
	4	26			
	20	34			
	100	45			
2	2	0	0		10
	5	4	0	0	
	10	51	63	48	
	20	95	88	90	
	50	100	100	92	
3	2	0	0	0	16
	5	2	13	0	
	10	22	70	0	
	20	77	69	0	
	50	100	100	84	
4	100	100	100	100	32
	2	0	0	0	
	5	3	0	0	
	10	7	0	0	
	20	28	32	26	
5	50	85	88	75	11
	100	100	100	100	
	2	0	0	0	
	5	5	0	0	
	20	92	100	82	
	50	100	100	100	
	100	100	100	100	

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Table 3. Inhibition of HIV by Phosphorothioate Oligonucleotides with Cholesteryl Substituents

compound	conc. µg/ml	inhibition				ID ₅₀ (syncytia)
		syncytia	P17	P24	RT	
6 (phospho- diester control)	2.5	15	13	22	33	6.0
	6.25	56	67	81	70	
	10	90	89	85	85	
	25	100	100	100	100	
7	0.25	0	12	19	23	0.8
	1.0	74	69	70	68	
	1.5	100	100	100	100	
	6.0	100	100	100	100	
8 (phospho- diester control)	1.6	0	0	0	0	14.5
	6.25	15	16	26	26	
	25	95	84	82	67	
	100	97	96	96	78	
9	1.6	23	39	43	47	3.2
	6.25	98	92	96	73	
	25	98	96	96	76	
	100	98	96	96	88	
10 (phospho- diester control)	1.6	0	0	0	0	>100
	6.25	0	4	4	0	
	25	0	20	22	25	
	100	0	24	33	28	
11	1.6	30	42	47	45	3.5
	6.25	97	86	88	61	
	25	97	92	92	70	
	100	98	92	96	89	
12	1.6	28	33	40		3.4
	6.25	93	60	67		
	25	100	100	100		
	100	100	100	100		
13	1.6	20	23	31		3.6
	6.25	89	56	67		
	25	100	100	100		
	100	100	100	100		
14	1.6	18	21	25		13
	6.25	35	30	32		
	25	90	70	66		
	100	100	100	100		
15	25	80	20	25		
	50	99	99	90		
	100	100	100	95		

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Table 4 New Modified Oligonucleotides

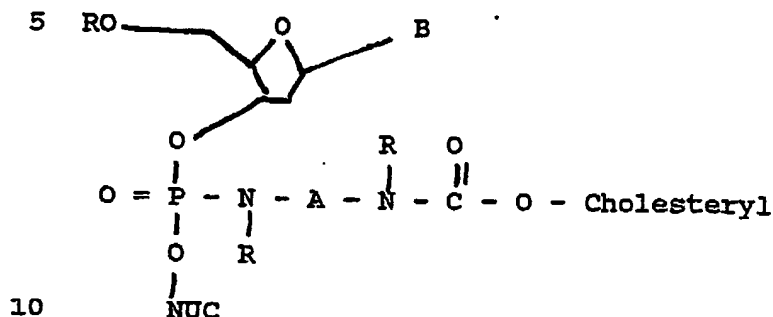
Compound	Structure	ID50 (ug/ml) ^a
1 ^b	AsCsAsCsCsCsAsAsTsTsCsTsGSAAsAsAsTsG _{chol} G	0.4-0.7
2	AsCsAsCsCsCsAsAsTsTsCsTsGSAAsAsA _{chol} TpG _{chol} G	1.7
3	GsTsGsTsCsTsCsCsCsCsTsTsCsTsTsCsCsTsGsCsGA _{chol} T	0.05
4	AsGsTsCsCsAsTsTsGsGsTsCsTsTsAsAsAsGsGsTsAsCsCsCsTsGsAsG _{chol} G	0.3
5	CholesterylpaCsAsCsCsCsAsAsTsTsCsTsGsAsAsAsAsTsGSG	1.5
6	CholesterylscTsGsAsAsAsAsTsGSG	7
7	CholesterylpcTsGsAsAsAsAsTsGSG	12
8	CsTsGsAsAsAsAsTsG _{chol} G	1.5-2.5
9	StigmasterylscTsTsGsAsAsAsAsTsGSG	7-14
10	TsTsTsTsTsTsTsTsT _{chol} T	10-18
11	TsTsTsTsTsTsTsTsT _{chol} T	15-30
12	CsCsCsCsCsCsCsCsC _{chol} C	6
13	CsCsCsCsCsCsCsCsC _{chol} C	7
14	CsCsCsCsCsCsCsC _{chol} C _{chol} C	1.7 (toxic > 4 ug/ml)

a. A range in activity is shown for compounds tested more than once.

b. This compound, reported previously, is included for reference.

What is claimed is:

1. An oligonucleotide comprising:



wherein A is selected from the groups consisting of an aliphatic alkyl, branched aliphatic alkyl and a heteroatom containing an alkyl (branched) chain of 2 to 18 carbon atoms, R is selected from the group consisting of H and lower alkyl up to 12 carbon atoms; NUC is selected from the group consisting of an oligonucleotide and a phosphothioate oligonucleotide and B is a base.

20

2. An oligonucleotide as set forth in claim 1 wherein said oligonucleotide includes 7 to 20 nucleotides.

3. A pharmaceutical composition consisting essentially of: an oligonucleotide and at least one steroid conjugated to said oligonucleotide for increasing the antiriral activity of said
5 oligonucleotide.

4. A composition as set forth in claim 3 wherein said steroid means is cholesterol conjugated to said oligonucleotide.
10

5. A composition as set forth in claim 4 wherein said oligonucleotides include phosphodiester bonds.

15 6. A composition as set forth in claim 5 including phosphothiate substitutions of phosphodiester bonds.

7. A composition as set forth in claim 3
20 wherein said oligonucleotide is complementary to a nucleotide region of a virus.

8. A composition as set forth in claim 7 wherein the virus is HIV-1 virus.
25

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9. A composition as set forth in claim 8 wherein said region of the HIV-1 virus is the TAT gene, the REV gene, or the NEF gene.

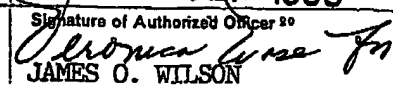
5 10. A composition as set forth in claim 3 wherein said steroid is stigmasteryl.

11. A composition as set forth in claim 3 including two steroids conjugated to said
10 oligonucleotide.

12. A composition as set forth in claim 3 wherein said oligonucleotide is a homopolymer.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/03204

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³ According to International Patent Classification (IPC) or to both National Classification and IPC INT CL. (5): A61K 31/00; C07H 15/00 U.S. CL: 514/45,46,49,50; 536/27,29		
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched ⁴</div> <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;">Classification System ¹</div> <div style="width: 45%;">Classification Symbols</div> </div> <div style="margin-top: 10px;"> U.S. 514/45,46,49,50; 536/27,29 </div> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin-top: 10px;">Documentation Searched other than Minimum Documentation to the extent that such documents are included in the fields searched ⁵</div>		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category ⁸	Citation of Document, ¹⁰ with indication, where appropriate, of the relevant passages ¹¹	Relevant to Claim No. ¹²
A,P	Proceedings of the National Academy of Science, Vol. 86, -- September 1989, Robert L. Letsinger et al., "Cholesterylconjugated oligonucleotides: Synthesis, properties, and activity as inhibitors of replication of human immunodeficiency virus in cell culture" pages 6553-6556.	1-12
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁵ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Δ" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ²		Date of Mailing of this International Search Report ³
28 AUGUST 1990		24 SEP 1990
International Searching Authority ¹		Signature of Authorized Officer ²⁰
RO/US		 JAMES O. WILSON

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